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(71)Applicant : SUMITOMO BAKELITE CO LTD

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(72)Inventor : YOKOYAMA KANEHISA
ASAI HIDEAKI

(54) FORMED COLLAGEN AND ITS PRODUCTION

(57)Abstract:

PURPOSE: To obtain a formed collagen having higher vibration durability and strength than collagen gel while holding almost the same structure as collagen gel, less changeable of the hardness during storage, keeping bio-compatibility characteristic inherent to collagen gel and suitable as a material for a culture medium, an artificial blood vessel, etc.

CONSTITUTION: An acidic collagen solution is added with an aqueous solution of an amino acid and a sugar, and a neutralizing solution to form collagen gel. This is brought into contact with a protein-crosslinking agent to react with each other. By the reaction, cross linkages are formed and the inner structure of the collagen is stabilized. Further, an aqueous solution of an inorganic salt is made to contact to remove useless components from the collagen gel. Subsequently, pure water is brought into contact to remove the inorganic salt by replacement.

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(71) 出願人 000002141

住友ベークライト株式会社
東京都品川区東品川2丁目5番8号

(72) 発明者 横山 兼久

秋田市土崎港相染町字中島下27-4 秋田
住友ベーク株式会社内

(72) 発明者 浅井 秀昭

秋田市土崎港相染町字中島下27-4 秋田
住友ベーク株式会社内

(54) 【発明の名称】 コラーゲン賦形物およびその製造方法

(57) 【要約】

【構成】 酸性コラーゲン水溶液に、アミノ酸や糖類の水溶液と中和液を加えてコラーゲンを生成させ、これに蛋白質架橋剤を接触、反応させて架橋化させ、コラーゲン内部の構造を保持させた。さらには、これに無機塩類の水溶液を接触させてコラーゲン中の不要な成分を置換除去し、次に純水を接触させて前記無機塩類を置換除去した。

【効果】 コラーゲンゲルとほぼ同等の構造を保ちながら、コラーゲンゲルに比べて振動耐久性や強度が高く、かつ保存中における硬さの変化も少なく、コラーゲンゲル本来の生体適合性を保持していて、培養用基材や人工血管等の素材として適している。

ラーゲンのゲル状素材のこのような欠点を解決しようとするもので、コラーゲンゲル表面や内部の構造を保持したまま、コラーゲンゲルの強度を向上させ、かつ作製ごとのコラーゲンゲル表面や内部の構造のばらつきが少ないコラーゲン賦形物を提供することにある。

【0006】

【課題を解決するための手段】本発明者らは、鋭意研究の結果、架橋剤を用いずに酸性コラーゲン水溶液の中和によりコラーゲンゲルを形成させた後、蛋白質の架橋剤を接触、反応させることにより、コラーゲンゲルの表面および内部の構造を保持し、コラーゲンゲルの強度を増強できること、さらに架橋剤を作用させるとき、コラーゲンゲル内部にアミノ酸類や糖類が含まれていると、架橋処理ごとの構造や特性のばらつきが小さいこと、および架橋剤による反応終了後、無機塩類を溶解させた溶液中に浸漬することにより、未反応の架橋剤や副生成物、その他アミノ酸や糖類を効率よく除去でき、得られたコラーゲン賦形物の経時的な変化が少ないことを見だし、本発明の完成に到った。

【0007】即ち本発明は、酸性コラーゲン水溶液に中和液を加えてコラーゲンゲルを生成させ、これに蛋白質架橋剤を接触、反応させて架橋化させ、コラーゲンゲル内部の構造を保持させたことを特徴とするコラーゲン賦形物であり、さらには、コラーゲンゲル構造中の未反応物および副生成物を置換除去し、水、両親媒性有機溶剤、もしくはこれらの混合液を含有させ、あるいは、これらを凍結乾燥したことを特徴とする。

【0008】またさらには、酸性コラーゲン水溶液に、ゲル調製成分としてのアミノ酸類や糖類の水溶液、および中和液を加えてコラーゲンゲルを生成させ、もしくは、コラーゲンゲル生成後に、ゲル調製成分を接触させてコラーゲンゲルの構造中に包含させた後、蛋白質架橋剤を接触、反応させて架橋化させ、コラーゲンゲル内部の構造を保持させ、これに無機塩類の水溶液を接触させて、コラーゲンゲル構造中のゲル調製成分、未反応物および副生成物を置換除去し、次に純水を接触させて前記無機塩類を置換除去することを特徴とするコラーゲン賦形物の製造方法である。

【0009】本発明は、架橋剤による処理の前に、コラーゲンゲルの構造を構築し、コラーゲンゲル内部に水を包含したまま架橋剤を反応させることにより、コラーゲンゲルの構造を保持した状態での架橋が可能であることと、コラーゲンゲルの内部と外部の濃度差による溶液の拡散により、即ちコラーゲンゲル内部に外部の溶液が侵入し、内部の溶液が外部の溶液中に拡散して、コラーゲンゲル内部に貯留した不要な成分を排出できることに基づく。

【0010】使用するコラーゲンとしては、I型コラーゲンなどコラーゲン単体でゲルを形成するものが選ばれる。コラーゲンゲルは静置の状態ではゲルの形状を保持

し、内部には水分を保持している。この状態で架橋剤と接解、反応させると、コラーゲン繊維間の距離が保たれ、コラーゲン繊維自身の架橋に留まるので、コラーゲンゲル内部の構造は保たれる。コラーゲンゲル中に保持されている水分中にアミノ酸や糖類が含有されていれば、過剰の架橋剤はこれらと反応し、架橋反応が適度に進むことがないので、反応時間や架橋剤の濃度による影響は少なくなり、架橋反応毎のばらつきは小さくなる。

【0011】架橋剤としては、一般には、蛋白質中のアミノ基やカルボキシル基と反応して架橋を起こすものが用いられる。そのような架橋剤としては、グルタルアルデヒド、ヘキサメチレンジイソシアネート、カルボジイミドや水溶性の多価エポキシ化合物などが挙げられるが、中でもグルタルアルデヒドやヘキサメチレンジイソシアネートが分子量が小さく、本発明においては、コラーゲンゲル内部の構造を壊す可能性が少なく適している。未反応のグルタルアルデヒドは細胞に対して毒性を有するが、無機塩類水溶液中への浸漬により容易に除去でき、毒性を示すことはなくなる。

【0012】架橋反応後は、コラーゲンゲル中に貯留した未反応の架橋剤や副生成物を除去する必要がある。コラーゲンゲルの内部は水の動きが遅く、純水に浸漬しただけでは、純水のコラーゲンゲル内部への拡散は起こりにくく、除去が難しい。特に、アミノ酸類や糖類などから成るゲル調製成分は、コラーゲンや水との親和性が高いため、コラーゲンゲルの構造中からの除去は非常に難しい。このような場合には、コラーゲンゲルを無機塩類等の高濃度の溶液と接触させて、溶液の濃度差を利用してコラーゲンゲル内に拡散させ、コラーゲン内部に貯留している不要な成分と置換させることにより、排出することが出来、効果がある。

【0013】この高濃度溶液の溶質は、コラーゲンゲル内部に進入して不要な成分を排出させた後、最終的にはそれ自身も水と交換して排出、除去されなければならない。このような処理を効率よく進めるためには、コラーゲンゲル内での移動が速やかに行われることが必要で、そのためには、分子量が小さく、かつ、溶液全体の電荷が中性であることが好ましく、そのような溶質としては無機塩類が最も適する。また、使用する無機塩類としては、金属イオンが沈殿を生じないものであることが必要であり、また毒性が低いことが望ましく、そのような金属としてはナトリウムやカリウムが挙げられる。陰イオンとしてはイオンの大きさが小さく動き易いほうが好ましく、これらを総合的にみると、塩化ナトリウムまたは塩化カリウムが使用する無機塩類として適当である。

【0014】また、コラーゲンゲルに接触させる水溶液は、コラーゲンゲル内部に速やかに浸入して、コラーゲンゲル内部に貯留しているゲル調製成分等を外部に拡散させる役目をするものであるから、濃度をなるべく高くし、濃度勾配をなるべく大きくするのが効率が高く、従

送時等の振動に対する耐久性の確認を行うため、実施例および比較例で得られたコラーゲン賦形物を、プレートごと振とう機にかけ、振幅 3 cm、1 0 0 回／分で 2 4 時間振動を与え、コラーゲン賦形物およびコラーゲンゲルの崩壊の状況を比較した。

【0026】〔保存における硬さの変化の比較〕室温 25℃において 6 ヶ月間保存し、コラーゲン賦形物およびコラーゲンゲルの硬さの変化を、調製直後の硬さと比較

した。実施例 2 については内部のエタノールを純水で置換し、また、実施例 3 については純水を加えてコラーゲン賦形物に水を包含させた状態で硬さを測定した。各々調製直後および調製後 6 ヶ月保存したものについて硬さを測定し、調製直後の比較例 2 のコラーゲンゲルの硬さを 1 0 0 として数値化した。

【0027】

【表 1】

表 1 コラーゲン賦形物の特性試験結果

	厚みの 比較試験	振動耐久性 試験(24時間)	保存試験(硬さの変化)	
			調製直後	6 ヶ月後
実施例 1	97	変化なし	310	320
・ 2	98	変化なし	300	305
・ 3	95	変化なし	330	330
比較例 1	75	崩壊、液状化	320	570
・ 2	100	変化なし	100	150

【0028】試験結果は表 1 に示した通りで、本発明によるコラーゲン賦形物は、厚みの減少が少なくコラーゲンゲルと同等の構造を保っているばかりでなく、振動を加えても崩解や変形を生ずることがなく、従来の方法で架橋させたコラーゲンゲル（比較例 1）に比較して耐久性に優れ、また保存によるコラーゲン賦形物の硬さの変化が少なく、経時的な架橋の進行が殆んどなく安定なことが明白である。

【0029】

【発明の効果】本発明のコラーゲン賦形物は、コラーゲンゲルとほぼ同等の構造を保ちながら、コラーゲンゲルに比べて振動耐久性や強度が高く、かつ保存中における変化も少なく、培養用基材や人工血管などの基材として、コラーゲンゲル本来の生体適合性を保持し、かつ、強度および保存性を高めた素材として好適である。

Fibril-forming Collagens in Lamprey*

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Joanne Kelly‡, Shizuko Tanaka‡, Thomas Hardt‡, Eric F. Eikenberry§, and Barbara Brodsky‡¶

From the ‡Department of Biochemistry and the §Department of Pathology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Five types of collagen with triple-helical regions approximately 300 nm in length were found in lamprey tissues which show characteristic D-periodic collagen fibrils. These collagens are members of the fibril-forming family of this primitive vertebrate. Lamprey collagens were characterized with respect to solubility, mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, carboxymethyl-cellulose chromatography, peptide digestion patterns, composition, susceptibility to vertebrate collagenase, thermal stability, and segment long spacing-banding pattern. Comparison with fibril-forming collagens in higher vertebrates (types I, II, III, V, and XI) identified three lamprey collagens as types II, V, and XI. Both lamprey dermis and major body wall collagens had properties similar to type I but not the typical heterotrimer composition. Dermis molecules had only $\alpha 1(I)$ -like chains, while body wall molecules had $\alpha 2(I)$ -like chains combined with chains resembling lamprey type II. Neither collagen exhibited the interchain disulfide linkages or solubility properties of type III. The conservation of fibril organization in type II/type XI tissues in contrast to the major developments in type I and type III tissues after the divergence of lamprey and higher vertebrates is consistent with these results. The presence of type II and type I-like molecules as major collagens and types V and XI as minor collagens in the lamprey, and the differential susceptibility of these molecules to vertebrate collagenase is analogous to the findings in higher vertebrates.

The family of collagen molecules in higher animals with uninterrupted (Gly-X-Y)_n triple-helical regions 300 nm in length and with the capacity to aggregate into fibrils with an axial period of D = 67 nm includes five distinct types of collagens: types I, II, III, V, and XI (1 α , 2 α , 3 α) (1-3). These five collagens have been designated the fibril-forming or group 1 collagens (1). Although these collagens show some sequence homology, especially in the distribution of charged residues (4-6), each genetic type has identifying characteristics, such as chain composition, amino acid composition, and the amount of hydroxylysine and hydroxylysine-linked disaccharides resulting from post-translational modification (7-9, see Table V). Group 1 collagens also differ in their susceptibility to vertebrate collagenase (10, 11): types I and III are cleaved rapidly and completely, while type II undergoes a slower, incomplete degradation and types V and XI are resistant to any cleavage.

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¶ To whom correspondence should be addressed.

Type I and II collagens are always major tissue components, while types V and XI constitute minor collagens in a tissue (1, 9). Type I is the predominant collagen in bone, tendon, and cornea, while type V is found as a minor component (usually less than 5%). Type II is the major collagen in cartilage, vitreous, and notochord, with type XI present in small amounts (less than 10%). Type III is found together with type I in skin, reticular tissues, and vascular tissues, where it constitutes 15-40% of total collagen.

Fibril-forming collagens evolved early in the development of multicellular animals. D-periodic fibrils containing molecules with a characteristic distribution of charged residues are found in many invertebrates (12-14). In the specialized tissues of vertebrates, a family of distinct fibril-forming types is seen. Lampreys, a member of the most primitive vertebrate class, have a collagen with $\alpha 1(I)$ -like chains in the dermis (15, 16), and have type II and XI collagens in the notochord (17, 18). Types I and II have been identified in sharks and bony fish (12, 19, 20), but it is only in avian and mammalian species that all five types (I, II, III, V, XI) have been reported (7, 8, 21, 22).

We report here the collagens present in three tissues of the lamprey which contain D-periodic collagen fibrils: the dermis, notochord, and body wall. Five types of collagen with triple-helical regions 300 nm in length were found. Of these, three types were identifiable as II, V, and XI by their solubility, chain composition, amino acid composition, and susceptibility to vertebrate collagenase. The two other lamprey collagen molecules did not correspond to types I and III of higher vertebrates in their chain composition.

MATERIALS AND METHODS

Extraction and Purification—Mature lampreys (*Petromyzon marinus*) were obtained from the New Hampshire Fish and Game Commission. Tissues were dissected and diced on ice and all procedures were done at 4 °C (unless otherwise stated). Acid extraction of dermis and pepsin extraction of notochord and subcutaneous tissues were carried out as previously described (17, 23). Salt fractionation at neutral pH was performed on collagen solutions in 1 M NaCl, 0.05 M Tris, pH 7.5, by bringing the NaCl concentration in steps to 1.7, 2.0, 2.6, and 5.0 M, with precipitates collected by centrifugation at 15,000 rpm for 1 h in a Sorvall SS-34 rotor. Differential salt fractionation at acid pH was performed by bringing collagen solutions in 0.5 M acetic acid to 0.9 M NaCl and then to 1.2 M NaCl, with precipitates collected by centrifugation as above (7).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)¹ was carried out as described (24, 25). Gels were stained with 0.25% Coomassie Blue, 10% trichloroacetic acid and destained with 7.5% acetic acid, 15% methanol. To reduce disulfide cross-links, 5% β -mercaptoethanol was added to the SDS sample buffer and the sample boiled for 3-4 min prior to electrophoresis.

¹ The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; SLS, segment long spacing; CMC, carboxymethylcellulose chromatography.

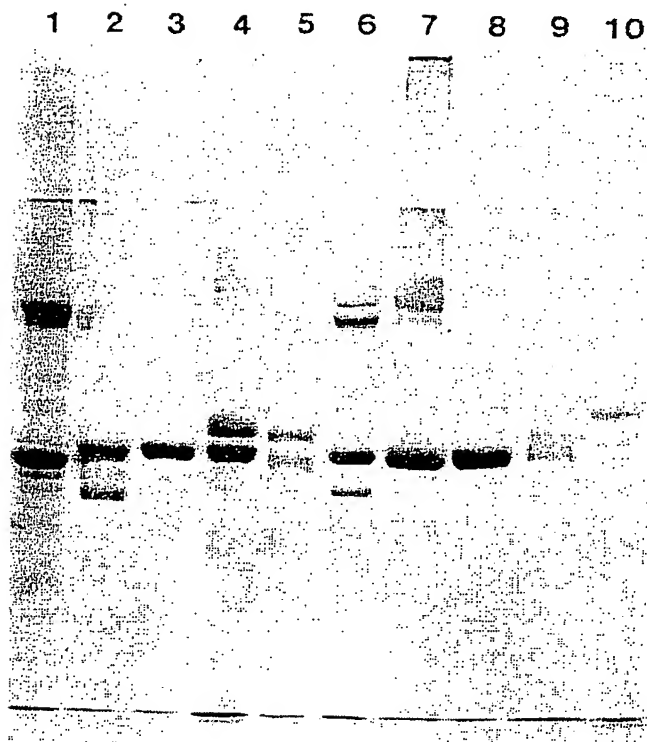


FIG. 2. SDS-PAGE (6% polyacrylamide gel) of five lamprey collagens and the five higher vertebrate group 1 collagens. Lane 1, lamprey dermis collagen; lane 2, lamprey body wall (subcutaneous) collagen; lane 3, lamprey notochord type II; lane 4, lamprey notochord minor collagen, type XI; lane 5, lamprey body wall minor collagen (type V); lane 6, rat tail tendon type I collagen; lane 7, calf type III collagen (reduced); lane 8, chick type II collagen; lane 9, chick type XI collagen; and lane 10, chick type V collagen.

lamprey collagens (below) indicated none contained inter-chain disulfide bonds.

Body Wall (or Subcutaneous) Collagen

The subcutaneous layer of lamprey skin joins the muscle layer and is continuous with tissue surrounding the spinal cord and notochord sheath. Collagen in this layer is not solubilized by acid or neutral salt solution but can be extracted by pepsin treatment. Pepsin extraction of subcutaneous tissue yielded one major collagen and a minor component. The major collagen had a solubility similar to type I, precipitating at 0.9 M NaCl at acid pH. During sequential salt fractionation at neutral pH, little collagen came out below 2.6 M NaCl (6%), with the majority precipitating at 2.6 M NaCl (47%) and 3.0 M NaCl (41%). On SDS-PAGE this collagen shows two bands running near $\alpha 1(I)$ which are often not resolved, and one band near $\alpha 2(I)$ (Fig. 2, lane 2). We denote these three chains as $\alpha 1$, $\alpha 1'$, and $\alpha 2$, respectively. A small amount of material was also seen in the β region.

On carboxymethyl-cellulose chromatography, $\alpha 1'$ eluted just after $\alpha 1$ (at a salt concentration near 0.03 M NaCl), while $\alpha 2$ eluted at a significantly higher salt concentration (0.065 M NaCl) (Fig. 6). The individual chains were isolated chromatographically and further characterized. The amino acid compositions of $\alpha 1$ and $\alpha 1'$ are similar (Table I). The CNBr and V-8 protease digestion patterns of $\alpha 1'$ chains which appeared pure on electrophoresis (Fig. 6, inset), showed the major bands present in $\alpha 1$ but also had some distinct bands

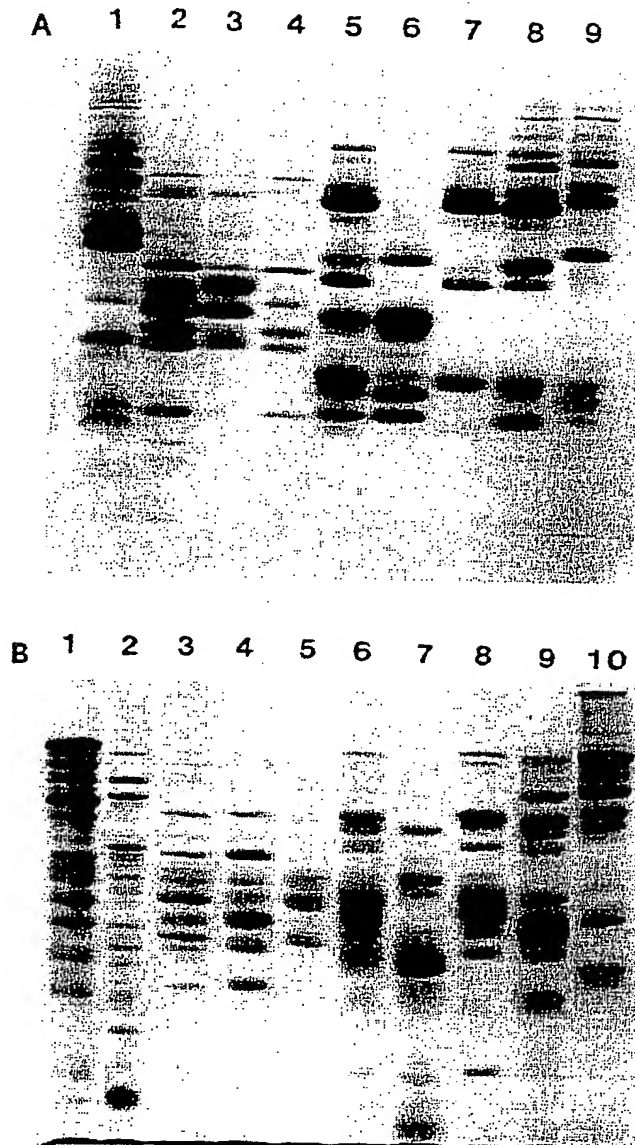


FIG. 3. SDS-PAGE of peptides after CNBr or V-8 protease digestion of isolated lamprey collagen chains. A, CNBr digestion products of: lane 1, chick $\alpha 1(I)$ standard; lane 2, lamprey dermis collagen; lane 3, lamprey dermis α ; lane 4, lamprey dermis β ; lane 5, lamprey body wall collagen; lane 6, body wall $\alpha 2$; lane 7, body wall $\alpha 1$; lane 8, body wall $\alpha 1'$; lane 9, lamprey notochord type II collagen. A 12.5% polyacrylamide gel was used for SDS-PAGE of the CNBr samples. B, V-8 protease digestion products of: lane 1, chick $\alpha 1(I)$ standard; lane 2, chick $\alpha 2(I)$ standard; lane 3, lamprey dermis collagen; lane 4, dermis α ; lane 5, dermis β ; lane 6, lamprey body wall collagen; lane 7, body wall $\alpha 2$; lane 8, body wall $\alpha 1$; lane 9, body wall $\alpha 1'$; lane 10, lamprey notochord type II. A 10% polyacrylamide gel was used for SDS-PAGE of the V-8 protease samples.

(Fig. 3A, lanes 7, and 8; Fig. 3B, lanes 8 and 9). These results suggest that $\alpha 1$ and $\alpha 1'$ are polypeptide chains related by degradation, variation in post-translational modification, or as products of duplicated genes. The amino acid compositions of the $\alpha 1$ and $\alpha 1'$ chains differ from lamprey dermis collagen and from $\alpha 1(I)$ chains of higher and lower vertebrates in their

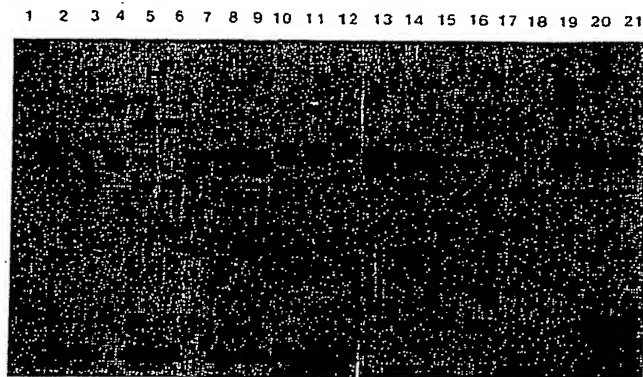


FIG. 4. SDS-PAGE with a 5–12% gradient gel showing vertebrate collagenase digestion of the five lamprey collagens. Rat tail tendon type I collagen was digested at 18 °C for 0, 6, and 30 h (lanes 1–3); lamprey dermis collagen was digested at 18 °C for 0, 6, and 30 h (lanes 4–6); the lamprey major body wall was digested at 18 °C for 0, 6, and 30 h (lanes 7–9); the minor component of the lamprey body wall tissue was digested at 18 °C for 0 and 30 h (lanes 10 and 11) and at 25 °C for 30 h (lane 12); chick type II collagen was digested at 25 °C for 0, 6, and 30 h (lanes 13–15); lamprey notochord type II was digested at 25 °C for 0, 6, and 30 h (lanes 16–18); and the lamprey notochord minor component (type XI) was digested at 25 °C for 0, 6, and 30 h (lanes 19–21).

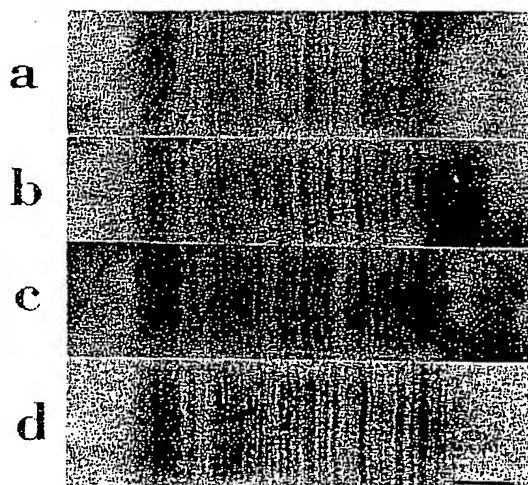


FIG. 5. SLS crystallite patterns of (a) lamprey body wall collagen; (b) lamprey dermis collagen; (c) lamprey notochord collagen; and (d) rat tail tendon type I collagen. The bar represents 50 nm.

collagen precipitated at 0.9 M NaCl in 0.5 M acetic acid, but a small amount of relatively pure minor collagen was soluble at this salt concentration and precipitated at 1.2 M NaCl. The chain with slower mobility could be separated on CMC (not shown), and its amino acid composition showed the low alanine and high hydrophobic amino acid contents of a type V collagen (Table IV, (5)) together with the lower hydroxyproline and higher serine expected for a primitive vertebrate (12). Studies of the faster moving band were hindered by contamination with the major $\alpha 1$ chain.

The major subcutaneous collagen underwent incomplete cleavage by human skin collagenase at a rate slower than that of type I and similar to that of type II collagen (Fig. 4, lanes 7–9). The minor component was not susceptible to

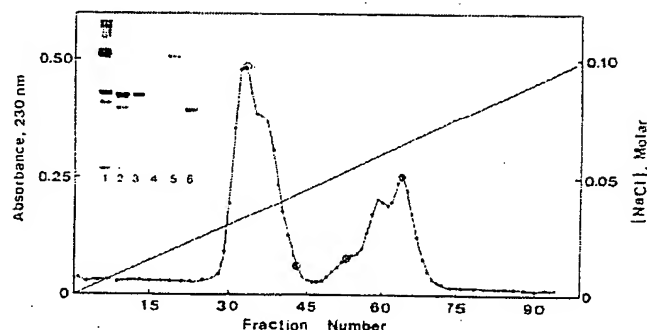


FIG. 6. Carboxymethyl-cellulose chromatography of the body wall major collagen. The column was equilibrated with 0.04 M sodium acetate containing 6 M urea, pH 4.8, at 42 °C and was eluted with an 0–0.10 M NaCl gradient. The $\alpha 1$ chains eluted at a lower salt concentration than $\alpha 2$ chains (0.032 M NaCl and 0.064 M NaCl, respectively). The inset shows SDS-PAGE (6% polyacrylamide gel) of selected fractions (circles on chromatogram): lane 1, rat tail tendon type I; lane 2, lamprey body wall collagen; lane 3, fraction 33; lane 4, fraction 43; lane 5, fraction 52; lane 6, fraction 64.

digestion by this enzyme, a property which is characteristic of type V collagen (Fig. 4, lanes 10–12). The major collagen had a sharp thermal transition near 28.5 °C, while the minor subcutaneous collagen melted near 32 °C, with a broader melting profile. The major subcutaneous collagen formed SLS with characteristic type I-banding patterns (Fig. 5). Although the minor collagen formed SLS crystallites, we were unable to obtain staining adequate for comparisons.

Notochord Collagen

Characterization of collagens in the notochord sheath of the lamprey was previously reported (17, 18). The major collagen was identified as type II, based on its electrophoretic mobility, solubility properties, and amino acid composition. We compared type II to other lamprey collagens, with respect to composition, CNBr peptide pattern, V-8 protease digestion patterns, and thermal stability (Fig. 3; and Table I). A minor collagen also present in notochord corresponds in its features to type XI collagen in higher vertebrate cartilage (Table IV).

Lamprey type II collagen was partially digested by human skin collagenase, at a rate similar to that seen for chick type II (Fig. 4), which is slower than that for mammalian and avian types I and III (10, 11). The minor notochord collagen was not digested, even after long time periods (Fig. 4, lanes 19–21). Both notochord collagens melted at 33 °C, showing broad melting transitions and both collagens showed SLS crystallite patterns similar to those seen for higher vertebrate types I and II collagens (Fig. 5).

DISCUSSION

Vertebrates first appeared in the fossil record about 500 million years ago. The earliest vertebrates (class Agnatha) were jawless fish with a notochord as the axial supporting structure (12), and lamprey and hagfish are contemporary survivors of this class. Lamprey tissues exhibiting D-periodic collagen fibrils were found to contain five collagen types with a pepsin-resistant triple-helical region of approximately 1000 residues as estimated from electrophoretic mobility on SDS-PAGE. These observations imply that at an early stage of vertebrate evolution the fibril-forming collagen family contained at least five members. The three major types form SLS crystallites with staining patterns indistinguishable from higher vertebrate type I. These observations support the hypothesis of a critical role for charge distribution and length

TABLE V
Higher vertebrate fibril-forming collagens and proposed related lamprey chains

Higher vertebrate genetic type	Chain	Hyl/chain ^a	Lamprey chain	Hyl/chain
I	$\alpha 1(I)$	5-9	Dermis α	6
	$\alpha 2(I)$	8-12	Body wall $\alpha 2$	11
II	$\alpha 1(II)$	20-24	Notochord $\alpha 1(II)$	22
III	$\alpha 1(III)$	5		
V	$\alpha 1(V)$	36	Body wall $\alpha 1$ minor	34
	$\alpha 2(V)$	23		
XI	$\alpha 1(XI)$	38	Notochord $\alpha 1(XI)$	31
	$\alpha 2(XI)$	40	Notochord $\alpha 2(XI)$	37
	$\alpha 3(XI)$	20	Notochord $\alpha 3(XI)$	22

^a Refs. 8 and 9.

amino acid composition (15), and its digestion by vertebrate collagenase. The molecular packing in lamprey dermis fibrils exhibits distinctive features found in mammalian skin fibrils,² including a shorter axial period near 65 nm (36). Kimura (15, 16) has suggested that the presence of two distinct chain types in dermis collagen supports its type I nature, but this molecule differs from type I in not being associated with a minor type V component and in lacking the usual heterotrimeric nature of type I since both of its chains are similar to $\alpha 1(I)$ rather than one chain having features of $\alpha 2(I)$. Features of dermis collagen are consistent with a relationship to type III collagen, including its presence in skin, its nonheterotrimeric nature, and its rapid digestion by vertebrate collagenase. However, the distinctive properties that distinguish type III from type I, such as disulfide bonding within the triple-helix and precipitation at 1.7 M NaCl at neutral pH (9), are not found in dermis collagen, suggesting these characteristics may have developed after the lamprey evolved. Alternatively, it is possible that type III collagen in lamprey is particularly sensitive to degradation and was not solubilized intact in our preparations.

Studies of dermis collagen led to the suggestion that identifiable $\alpha 2(I)$ chains did not evolve until after the divergence of the lamprey. However, we have identified a collagen type in the body wall which contains a chain with $\alpha 2(I)$ characteristics. The presence of this $\alpha 2(I)$ -like chain suggests that $\alpha 2(I)$ as seen in skins of sharks, fish, and all higher vertebrates did not evolve from one of the dermis chains (15, 16) but was already present in recognizable form in the lamprey. In addition to its heterotrimeric nature, the major body wall collagen resembles type I in being widely distributed in different tissues, in being associated with a minor type V-like collagen, and in the similarity of its axial and lateral molecular packing to that seen in fibrils of bone and some tendons.² However, its $\alpha 1$ and $\alpha 1'$ chains are distinct from $\alpha 1(I)$ in amino acid composition and resemble lamprey type II chains in terms of composition, V-8 protease, and CNBr peptide patterns and digestion by vertebrate collagenase. Thus, dermis collagen and body wall collagen both have some characteristics resembling type I collagen, but neither has a traditional chain composition, since the dermis molecule contains only $\alpha 1(I)$ -like chains while the body wall molecule contains an $\alpha 2(I)$ -like chain in the same triple-helix as a chain different from $\alpha 1(I)$.

Homology between the sequences of $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(II)$, $\alpha 1(III)$, and $\alpha 2(V)$ suggest these chains diverged at least 500

million years ago (37, 38). Our finding of $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(II)$, $\alpha 1(V)$ (or $\alpha 2(V)$), $\alpha 1(XI)$, $\alpha 2(XI)$, and $\alpha 3(XI)$ chains in the lamprey supports an early divergence of group 1 chains. The chains of types II, XI, and V are found grouped in molecules similar to those found in higher vertebrates, but no molecules were found with a composition corresponding to type I. Our data raise the possibility that although the group 1 polypeptide chains may have diverged and been present at the time vertebrates evolved, type I chains may not have been present in the same triple-helices we find today. The presence in shark skin of a type I collagen molecule consisting of an $\alpha 2(I)$ chain closely related to the α chain in lamprey dermis and an $\alpha 2(I)$ chain closely resembling the $\alpha 2$ chain of lamprey body wall raises the possibility that after the divergence of lamprey and higher vertebrates these chains may have joined to form the type I molecule of higher vertebrates (Table V). Such an association would require changes in the C-propeptides which are thought to govern chain selection (1).

The presence of type II collagen in notochord and type I-related molecules in the dermis and body wall suggests that different genetic types were involved in tissue specialization in early vertebrates. The status of type II and type I-related molecules as major collagenous components and types V and XI as minor components was apparently established by the time the lamprey evolved and has been maintained in modern lampreys and mammals. Collagen molecules with amino acid compositions similar to type V are found as the major component in some invertebrate tissues with D-periodic fibrils (39), suggesting that relegation of type V to a minor status may have occurred as vertebrates developed. No lamprey collagen occupies the higher vertebrate type III niche, where type III comprises 15-40% of total collagen in specific tissues such as skin.

The degree of susceptibility of the five lamprey collagens to vertebrate collagenase digestion was a useful property to compare with mammalian collagen types. The resistance of the minor type V and XI collagens to cleavage and the digestibility of the three major lamprey collagens is strikingly similar to the situation in mammals and may relate to mechanisms of degradation established early in vertebrate evolution. We believe our data characterize the lowest vertebrate collagens known to be cleaved by vertebrate collagenase.

The data on lamprey and higher vertebrate collagens reflects proteins that have evolved separately for over 500 million years. We believe that the structure of lamprey collagens closely reflects early vertebrate forms since the body morphology of the lamprey today is similar to that seen in fossils from 300 million years ago (40). During the development of higher vertebrates from their primitive ancestors, there have been major changes in body morphology and connective tissues, with the appearance of bone and tendon (with largely type I collagen) and marked changes in collagen organization in skin. The conservation of fibril diameter, molecular packing, and morphology seen for type II fibrils throughout the vertebrates is consistent with the conservation of type II molecular features. In contrast, types I and III collagens have undergone substantial evolution in molecular composition since early vertebrates, and this is consistent with the dramatic changes in tissues containing these types.

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